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Original Article

Microbial composition of dental plaque microflora on first molars with orthodontic bands and brackets, and the acidogenic potential of these bacteria

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ABSTRACT

Purpose: In order to elucidate the characteristics of orthodontic appliance-associated dental plaque, this study aimed to profile the microflora and to estimate the acidogenic potential of supragingival plaque on first molars with orthodontic bands or brackets or without appliances.
Material and methods: Supragingival plaque samples from the surface of upper and lower first molars with orthodontic bands or brackets or without appliances in 6 subjects (age, 11–30 years) were cultured anaerobically on blood agar plates. Isolated bacteria were identified by 16S rRNA sequencing. The acidogenicity of isolated bacteria was examined using fastidious anaerobe agar plates containing bromocresol.
Results: Bacterial growth (log CFU/mg) was 6.6 ± 6.5, 6.9 ± 7.1, and 7.4 ± 7.6 in samples obtained from molars with bands, brackets, and without appliances, respectively. *Actinomyces* (43.5 and 40.0%) and *Streptococcus* (23.5 and 34.7%) were the predominant species present on molars with orthodontic brackets and without appliances, respectively. In contrast, the proportion of *Streptococcus* (44.4%) was higher than that of *Actinomyces* (17.6%) on molars with orthodontic bands ($P < 0.01$). The proportions of acidogenic bacteria in plaque samples from molars with bands, brackets, and without appliances were 74.5%, 71.3%, and 81.6%, respectively, although these differences were not statistically significant.
Discussion and conclusion: These results indicate that there are differences in the microbial composition and acidogenic potential of supragingival plaque from first molars with bands, brackets, or without appliances, and suggest that supragingival plaque on teeth with brackets carries predominantly periodontitis-associated bacteria but less caries-associated bacteria.

1. Introduction

More than 600 bacterial species comprise the plaque microflora that exists on surfaces within the oral cavity [1,2]. Numerous environmental changes within the oral cavity can lead to changes in the composition of plaque microflora [3].

The risk of oral diseases, including dental caries, gingivitis, and periodontitis, increases when various orthodontic appliances are attached to the enamel surface of teeth [4]. For instance, the risk of dental caries is reported to increase as the proportion of caries-related bacteria increases in the plaque microflora [5]. Caries-related bacteria, including *Streptococcus mutans* and *Lactobacillus* [6–10], and periodontitis-associated bacteria, such as *Eubacterium, Fusobacterium,* and *Treponema* [10–16], have been detected in orthodontic appliance-associated dental plaque by PCR and other molecular techniques. However, with the use of such molecular techniques alone, it is not only difficult to clarify the microbial composition of plaque microflora but also to determine the amount of live bacteria within the microflora. To date, no comprehensive studies have been carried out where anaerobic culture is used in combination with 16S rRNA gene sequencing for the accurate comparison of bacteria in plaque microflora associated with orthodontic appliances. Furthermore, despite the fact that plaque biofilm is considered to carry an increased risk of cariogenicity [17–19], we were unable to find any previous studies that have used bacterial microflora isolates to determine its acid productivity in patients with orthodontic attachments.

Therefore, the aim of this study was to determine the bacterial levels, composition, and acidogenic potential of plaque microflora on upper and lower first molars without appliances or with bands or brackets, in order to elucidate the characteristics of orthodontic appliance-associated dental plaque.

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2. Materials and Methods

2.1. Subjects

Six periodontally healthy subjects (4 females and 2 males; aged 11–30 y; mean, 21.5 y) undergoing orthodontic treatment at the Clinical Division of Orthodontics, Tohoku University Hospital, Sendai, Japan, were randomly selected for this study. Subjects were medically healthy, had received no antibiotics for 3 weeks before sampling, and were not taking immunosuppressive medication, anti-inflammatory drugs, or radiation therapy. Furthermore, they had no anamnesis of pregnancy, genetic diseases, or smoking habits. There were no instances of gingival sulcus depth greater than 3 mm or alveolar bone loss. Informed consent was obtained from all subjects, and the study was approved by the Ethics Review Board of Tohoku University, Graduate School of Dentistry, Sendai, Japan.

2.2. Orthodontic bands and brackets

Orthodontic bands (Tomy, Tokyo, Japan) made of stainless steel (sterilized) were fixed on the first molars using self-curing, glass ionomer cement (Fuji IX, GC, Tokyo, Japan). A dental probe and scaler were used for cleaning residual cement from the bands. Orthodontic brackets (Dentsply-Sankin, Tokyo, Japan) made of stainless steel (sterilized) were fixed on the surface of first molars using self-curing, adhesive resin (Super Bond, Sun Medical, Moriyama, Japan). The average sampling periods after fixation of orthodontic bands and brackets were 3.8 months (range, 2.6–5.0 months) and 3.1 months (range, 0.5–9.3 months), respectively (Table 1). Until the time of sampling, subjects were recalled once a month and were given oral hygiene instructions in order to ensure adequate tooth brushing technique. Subjects did not use mouth rinses (e.g., chlorhexidine) during the trial period, but there were no restrictions with regard to diet. Subjects were asked to refrain from tooth brushing on the morning before plaque sampling.

2.3. Sampling procedure

After sampling sites were isolated with cotton rolls, supragingival plaque samples (buccal sites) on the upper or lower first molars with orthodontic bands or brackets (on the tooth surface between the area above gingival margin and below the bands or brackets) were collected with sterilized toothpicks. Control samples from first molars without orthodontic bands or brackets were obtained at the same time as the test samples in subjects 1, 5, and 6. In addition, control samples were obtained from subject 3 one month before fixation of orthodontic bands and brackets, and from subjects 2 and 4 one month after the removal of orthodontic brackets.

2.4. Isolation of bacteria

Samples were transported in tightly screw-capped tubes, weighed, and transferred as soon as possible to an anaerobic glove box (Model AZ-Hard, Hirasawa, Tokyo, Japan) containing 80% N_2, 10% H_2, and 10% CO_2. Each sample was then suspended at a concentration of 1.0 mg/mL in sterilized 40 mM potassium phosphate buffer (pH 7.0) and was dispersed with a Teflon homogenizer. Serial 10-fold dilutions (0.1 mL each) were spread onto the surface of CDC anaerobic 5% sheep blood agar (BD, Franklin Lakes, NJ) plates, and were incubated in the anaerobic glove box at 37 °C for 7 d. After incubation, colony-forming units (CFU) on the plates were enumerated, and all colonies from plates having fewer than 100 colonies (mean, 16.5; range, 4–24

Table 1
Clinical features of subjects and bacterial growth in samples.

Subjects	Orthodontic bands								Orthodontic brackets								Without appliances							
	1	3	5	6				Mean ± SD	2			3		4			Mean ± SD	1	2			3		4		5	6		Mean ± SD
Age (years)	26	20	11	30				21.8 ± 8.3	25			20		17			20.7 ± 4.0	26	25			20		17		11	30		19.5 ± 6.9
Gender	Female	Male	Male	female					Female			Male		Female				Female	Female			Male		Female		Male	Female		
Sampled tooth (first molar)[a]	UR	UL	UR	UL	UR	UL			UR	UL	LR	UL	LR	UR	UL	LR		LR	UR	UL	LR	UR	UL	LR	LL	LR	LR		
Sampling period[b]	5.0	5.0	3.0	3.0	4.0	4.0		3.8 ± 1.0	0.7	0.7	0.5	0.7		9.3	8.0	2.0	3.1 ± 3.8	I [c]	II [c]			III [c]		II [c]		I [c]	I [c]		
Log (CFU/mg)	6.7	6.7	6.3	7.0	6.7	6.2		6.6 ± 6.5	6.5	6.5	6.5	6.2		7.6	6.8	6.8	6.9 ± 7.1	6.5	5.9	6.5	7.7	7.3	6.6	6.8	8.2	7.3	6.8	6.5		7.4 ± 7.6

[a] UR, upper right; UL, upper left; LR, lower right; LL, lower left.
[b] Sampling period (months) after attachment of orthodontic appliances.
[c] I, concurrently; II, 1 month after removal; III, 1 month before application.

colonies) were sub-cultured onto CDC plates. All plates, media, buffer solutions, and experimental instruments were kept in the anaerobic glove box for at least 24 h before use. To ensure strictly anaerobic conditions in the glove box, the reduction of methyl viologen (−446 mV) was carefully monitored whenever experimental procedures were carried out.

2.5. DNA extraction and identification of bacteria by 16S rRNA gene sequencing

Genomic DNA was extracted from each single colony using InstaGene Matrix kits (BioRad Laboratories, Richmond, CA), in accordance with the manufacturer's instructions.

The 16S rRNA gene sequences were amplified by PCR using universal primers 27 F and 1492 R [20,21] and *Taq* DNA polymerase (HotStar *Taq* Master Mix, Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Primer sequences were as follows: 27 F, 5′-AGA GTT TGA TCM TGG CTC AG-3′ and 1492 R, 5′-TAC GGY TAC CTT GTT ACG ACT T-3′. Amplification proceeded using a PCR Thermal Cycler MP (Takara Biomedicals, Ohtsu, Japan) programmed as follows: 15 min at 95 °C for initial heat activation; 30 cycles of 1 min at 94 °C for denaturation, 1 min at 55 °C for annealing, and 1.5 min at 72 °C for extension; and 10 min at 72 °C for final extension. PCR products were separated on 1% agarose gels (High Strength Analytical Grade Agarose, BioRad Laboratories) in Tris-borate EDTA buffer (100 mM Tris, 90 mM borate, 1 mM EDTA; pH 8.4), stained with ethidium bromide and photographed under UV light, and their sizes (ca. 1466 bp) were compared with molecular weight markers (100-bp DNA Ladder; Invitrogen Corp., Carlsbad, CA). PCR products were purified using the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare UK Ltd., Buckinghamshire, UK), followed by sequencing at Hokkaido System Science Co. Ltd. (Sapporo, Japan), using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems Japan Ltd., Tokyo, Japan) and an automated DNA sequencer (PRISM-3100, Applied Biosystems Japan Ltd.). Primer 1492 R was used for sequencing (at least 700 bp), and partial 16S rRNA gene sequences were then compared with 16S rRNA gene sequences from the GenBank database using the BLAST search program through the National Center for Biotechnology Information website. Bacterial species were determined by percent sequence similarity (> 97%).

2.6. Determination of bacterial acidogenicity

In order to determine their acidogenicity, bacterial isolates obtained above were cultured on Fastidious Anaerobe Agar (FAA, Lab M, Bury, UK) plates, containing 0.004% bromocresol purple [22] in the anaerobic glove box at 37 °C for 2 d. After incubation, bacteria forming yellow (< pH 5.2) zones around their colonies on the plates were termed acidogenic bacteria. On the other hand, those forming purple (> pH 6.8) zones on the plates were termed non-acidogenic bacteria. Furthermore, the acidogenicity of bacterial isolates was confirmed by collecting 1 loopful of each bacterial colony from the CDC anaerobe blood agar plate, followed by culture in 1.0 mL of 1.7% tryptone broth, containing 1.0% glucose in the anaerobic glove box at 37 °C for 2 d. After incubation, pH values of the culture suspensions were measured with a pH meter (pH BOY, Shindengen Electric Manufacturing Co., Ltd., Tokyo, Japan), and were confirmed to be < pH 5.2.

2.7. Data analysis

Tukey's tests were used to analyze differences between data, with P values < 0.05 being considered to be statistically significant.

3. Results

Bacterial growth (log CFU/mg) in plaque samples from molars with orthodontic bands, brackets, and without appliances was 6.6 ± 6.5, 6.9 ± 7.1, and 7.4 ± 7.6, respectively (Table 1). The proportions of facultative anaerobes from molars with orthodontic bands, brackets, and without appliances were 83.3%, 78.3%, and 83.7%, respectively (Table 2). Among the facultative anaerobes, the proportion of *Streptococcus* (44.4%) was higher than that of *Actinomyces* (17.6%) in samples from molars with orthodontic bands ($P < 0.01$). In contrast, *Actinomyces* (43.5 and 40.0%) and *Streptococcus* (23.5 and 34.7%) were more predominant in plaque from molars with orthodontic brackets and without appliances, respectively. Furthermore, the facultative anaerobe, *Neisseria,* was predominant in the plaque microflora from molars with orthodontic bands (Table 2).

Among the obligate anaerobes, *Veillonella* was predominant on molars with orthodontic brackets (8.7%) and without appliances (10.0%), but was scarcely detected on molars with orthodontic bands (1.9%) (Table 2). *Eubacterium* and *Porphyromonas* were specifically detected on molars with orthodontic brackets, but not on those with orthodontic bands and without appliances.

The proportions of acidogenic bacteria were highest on the molars without appliances (81.6%), followed by those with orthodontic bands (74.5%) and brackets (71.3%) (Table 2). The final pH values for culture media containing acidogenic bacteria from molars with orthodontic bands, brackets, and without appliances were 4.5 ± 0.2, 4.3 ± 0.2, and 4.3 ± 0.1, respectively. Most of the acidogenic bacteria were facultative anaerobes, such as *Streptococcus* and *Actinomyces*. The total proportions of *Streptococcus* and *Actinomyces* among acidogenic bacteria were 83.8% (67 of 80 strains), 93.9% (77 of 82), and 91.6% (142 of 155) in samples from molars with orthodontic bands, brackets, and without appliances, respectively. The other acidogenic facultative anaerobes were *Gemella*, *Haemophilus,* and *Actinobacillus*. In contrast, acidogenic obligate anaerobes were *Atopobium* and *Prevotella* (Table 2). There were no particular relationships between sampling period (e.g., before fixation or after removal of orthodontic appliances) and microbial composition.

4. Discussion

Bacterial growth on molars with orthodontic bands was slightly lower than on those with brackets and without appliances (Table 1). Ionomeric materials, particularly glass ionomer cement, have shown antimicrobial properties, which have been attributed to their ability to release fluoride [23,24]. Furthermore, there are other elements that are simultaneously released from glass ionomer cement [25], and these elements may have effects on plaque microflora adjacent to orthodontic bands.

In the present study, the composition of plaque microflora differed depending on surface properties. More specifically, in accordance with a previous study on the composition of plaque microflora on enamel, we found the predominant bacteria in plaque microflora on the surface of molars with orthodontic brackets and without appliances to be *Actinomyces, Streptococcus,* and *Veillonella* [26]. In contrast, in the plaque microflora from molars with orthodontic bands, the proportion of *Streptococcus* was higher, while that of *Actinomyces* and *Veillonella* was lower (Table 2).

In the current study, the proportion of acidogenic bacteria was highest on molars without appliances (81.6%), followed by those with orthodontic bands (74.5%) and brackets (71.3%) (Table 2). Most of the acidogenic bacteria were facultative anaerobes, such as *Streptococcus* (non-mutans streptococci, data not shown) and *Actinomyces*. It is known that non-mutans streptococci and

Table 2
Bacterial composition of plaque biofilm on molars with orthodontic bands, brackets and without appliances.

	Orthodontic bands								Orthodontic brackets								Without appliances											
Subjects	1	1	3	5	5	6	6	Total	2	2	2	3	4	4	4	Total	1	2	2	2	3	3	3	4	4	5	6	Total
Sampled tooth (first molar)[a]	UR	UL	UL	UR	UL	UR	UL		UR	UL	LR	UL	UR	UL	LR		LR	UR	UL	LR	UR	UL	LL	LR	LL	LR	LR	
Total isolates	16	5	17	17	21	17	15	108 (100)[b]	4	18	21	13	21	23	15	115 (100)	11	11	17	21	17	11	18	24	21	24	15	190 (100)
Total of acidogenic bacterial isolates[c]	4	2	16	15	14	15	14	80 (74.5)	2	8	12	12	18	19	11	82 (71.3)	8	11	17	20	10	7	7	19	20	23	13	155 (81.6)
Facultative anaerobes	15	5	11	16	14	15	14	90 (83.3)	3	11	15	12	17	18	14	90 (78.3)	9	11	17	20	11	8	8	19	19	24	13	159 (83.7)
Streptococcus[c]	2	2	10	6	10	10	8	48 (44.4)[d]	2	2	9	6	4	1	3	27 (23.5)	4	10	11	4	7	2	2	5	6	7	8	66 (34.7)
Actinomyces[c]			1	7	1	4	6	19 (17.6)[e]		4	3	6	13	17	7	50 (43.5)			4	16	3	4	5	13	12	16	3	76 (40.0)
Neisseria	9	3						12 (11.1)			1					1 (0.9)							1					1 (0.5)
Gemella[c]	1			2	3	1		7 (6.5)		1					1	2 (1.7)	1	1	2						1	1	2	8 (4.2)
Capnocytophaga	1			1				2 (1.9)	1	3	1				1	6 (5.2)	1				1			1				3 (1.6)
Haemophilus[c]	1							1 (0.9)								0	2											2 (1.1)
Rothia	1							1 (0.9)								0						1						1 (0.5)
Actinobacillus[c]								0								0	1											1 (0.5)
Campylobacter								0		1	1					2 (1.7)												0
Kingella								0							2	2 (1.7)												0
Obligate anaerobes	1	0	6	1	7	2	1	18 (16.7)	1	7	6	1	4	5	1	25 (21.7)	2	0	0	1	6	3	10	5	2	0	2	31 (16.3)
Atopobium[c]			5					5 (4.6)						1		1 (0.9)												0
Peptostreptococcus				1	3		1	5 (4.6)						2	1	3 (2.6)												0
Slackia					3			3 (2.8)								0												0
Veillonella					1	1		2 (1.9)	1	2	5	1	1			10 (8.7)	1				3		10	2	1		2	19 (10.0)
Cardiobacterium						1		1 (0.9)								0				1								1 (0.5)
Leptotrichia	1							1 (0.9)						1		1 (0.9)	1				1							2 (1.1)
Olsenella			1					1 (0.9)								0												0
Eubacterium								0					2	1		3 (2.6)												0
Fusobacterium								0		1						1 (0.9)						1		1				2 (1.1)
Porphyromonas								0		2	1					3 (2.6)												0
Prevotella[c]								0		1			1			2 (1.7)								1	1			2 (1.1)
Selenomonas								0		1						1 (0.9)					2	2		1				5 (2.6)

[a] UR, upper right; UL, upper left; LR, lower right; LL, lower left.
[b] Percentages are given in parentheses.
[c] After two-day incubation, bacteria forming yellow (< pH 5.2) zones around their colonies on fastidious anaerobe agar plates containing 0.004% bromocresol purple were termed acidogenic bacteria.
[d] Significantly different ($P < 0.01$) between e.
[e] Significantly different ($P < 0.01$) between d.

Actinomyces are the initial colonizers in plaque biofilm formation [27]. These findings suggest that the acidogenic potential of plaque microflora upon fixation of orthodontic brackets and/or bands is unexpectedly lower than that of teeth without appliances. Rather, periodontitis-associated bacteria may be more likely to colonize the former plaque microflora (see below).

In the plaque microflora from molars with orthodontic bands, the facultative anaerobe, *Neisseria,* was predominant (Table 2). In contrast, in microflora from molars with brackets or without any orthodontic appliances, the obligate anaerobe, *Veillonella*, known as a late colonizer in dental plaque formation, was predominant. It has been reported that *Neisseria* species colonize and constitute 1.3% to 15.2% of the early plaque biofilm on enamel [28]. These findings suggest that the partial pressure of oxygen may be higher in plaque that forms close to metal, possibly due to the plaque structure being less mature [29]. In turn, this would result in the low proportions of obligate anaerobes found in plaque on molars with orthodontic bands (Table 2), similar to that reported in shallow periodontal pockets [30].

Eubacterium, *Porphyromonas*, *Fusobacterium,* and *Prevotella* were detected in the plaque on molars with orthodontic brackets, as well as from those without appliances (Table 2), but were not detected on molars with orthodontic bands. It has been reported that these obligate anaerobes are one of the components of mature plaque microflora [2,31,32], and that they are associated with periodontitis [33,34]. In addition, *Prevotella intermedia* and *Tannerella forsythia* have frequently been reported to be present in subgingival plaque after fixation of orthodontic brackets [13,35]. Furthermore, the CFU ratio of anaerobic bacteria has been reported to increase after fixation of orthodontic appliances [36–39]. These findings suggest that plaque microflora on molars with orthodontic brackets is similar to that of periodontitis-associated microflora. Due to the complex structure of brackets, it is possible that oxygen scarcely infiltrates into dental plaque on the surfaces of teeth with orthodontic brackets. Thus, establishment of anaerobic environments in this way can promote obligate anaerobes, including periodontitis-associated bacteria, such as *Eubacterium*, *Porphyromonas*, *Fusobacterium*, and *Prevotella*, to colonize and grow [3,30,40].

We found no particular relationship between sampling sites (e.g., upper and lower first molars) and microbial composition. Furthermore, since relatively few plaque samples were examined in this study, correlations between age and ecologic distribution of oral microbiota remain uncertain, and further large-scale studies are necessary.

5. Conclusions

The findings of the present study indicate that there are differences in the microbial composition and acidogenic potential of supragingival plaque on first molars with orthodontic bands, brackets, and without appliances. Our data further suggest that supragingival plaque on teeth with brackets carries higher levels of periodontitis-associated bacteria but less caries-associated bacteria.

Conflict of interest

No potential conflicts of interest are disclosed.

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